Immunological Characterization of Membrane Skeletal Proteins in *Tetrahymena pyriformis* GL

A Thesis Presented to

The College of Arts and Sciences

Drake University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Brian T. Andrews

June 1998

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ABSTRACT

The cell cortex (or pellicle) of *Tetrahymena* is composed of three closely associated layers: the cell membrane, the alveolar sacs, and the epiplasm. The epiplasm is the innermost layer of the cortex and is thought to be analogous to the membrane skeleton found in eukaryotic cells, since it consists largely of filamentous proteins responsible for maintaining cell stability and shape. Furthermore, the epiplasmic layer provides a means by which the cell can regulate the location and distribution of structures along its surface.

Low salt extraction of Triton-potassium iodide (TKI) cortical residues is an efficient means by which three membrane skeletal proteins (band A (235 kDa), band C (125 kDa), and a 23 kDa protein) can be isolated. Previous immunofluorescence studies have localized bands A and C in the cell cortex and immunoblots have demonstrated that these proteins are major components of the membrane skeleton in *Tetrahymena.*

In this study, polyclonal antibodies were raised in rabbits against three membrane skeletal proteins isolated by low salt extraction of TKI cortical residues: band A and C, and a 23 kDa protein. Immunoblots were performed to characterize the number and size of immunoreactive proteins in total cellular protein as opposed to previous studies which only probed cortical proteins. Furthermore, immunoblots were performed to test the specificity of each antisera prepared against specific proteins in an attempt to clarify apparently contradictory data obtained by previous studies using both polyclonal and monoclonal antibodies.

Polyclonal antibodies against band A did not detect the presence of band A at 235 kDa, but did detect three bands between 100 and 140 kDa. Affinity-purified band C antibodies were demonstrated to identify a 125 kDa protein from whole-cell lysates, and no bands above 125 kDa, as previous studies have recognized. Interestingly, the 23 kDa polyclonal antibody recognized not only a 23 kDa protein from the total cellular protein, but a high molecular weight protein (above 125 kDa) as well.

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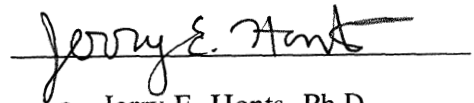
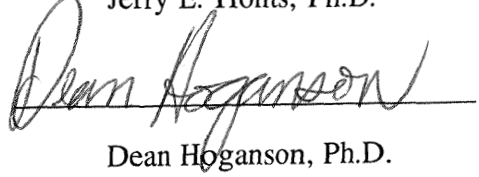
Approved by Committee



Jerry E. Honts, Ph.D.



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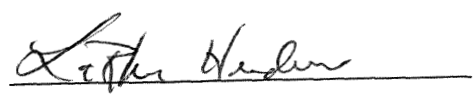

LaRhee Henderson, Ph.D.

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INTRODUCTION

The cell cortex (or pellicle) of *Tetrahymena pyriformis* GL is composed of three closely associated layers, as shown in Fig. 1 (derived from Allen, 1967). The cell membrane makes up the outermost layer of the pellicle (Williams, 1990). Underneath the cell membrane lies a continuous network of alveolar sacs which are thought to be analogous to the sarcoplasmic reticulum, found in muscle and nerve cells, by serving as a Ca^{++} storage organelle (Satir & Wissig, 1982). The innermost layer is the epiplasm and is found between the alveolar sacs and the cytoplasm (Fraúre-Fremíet, 1962).

The epiplasm is analogous in function to the membrane skeleton found in eukaryotic cells. It consists of a fibrillar network of proteins and is important in maintaining cell stability and shape (Vaudaux, 1976) as well as controlling the distribution and location of structures along the cell's surface. This layer is capable of maintaining the shape of the cell even after the cell membrane and alveolar sacs have been removed (Vaudaux *et al.* 1977). Williams *et al.* (1990) demonstrated that Triton-high salt (THS) extracts of *Tetrahymena* cells produced cell "ghosts" consisting of microtubule-depleted cortical residues as seen by light microscopy observations. The fibrillar protein network of the epiplasm in some ways resembles the spectrin network found on the inner surface of erythrocyte membranes (Williams *et al.* 1979).

In *Tetrahymena pyriformis* GL, Triton-high salt (THS) extracts of the cell cortex have been shown to be composed largely of three high molecular weight polypeptides (besides tubulin and other proteins) with molecular weights of 235, 145, and 125 kDa (Vaudaux, 1976). These proteins were named band A, band B, and band C respectively. Low salt extracts of Triton-potassium iodide (TKI) cortical residues reveal the presence of the same three membrane skeletal proteins as well as substantial quantities of a low

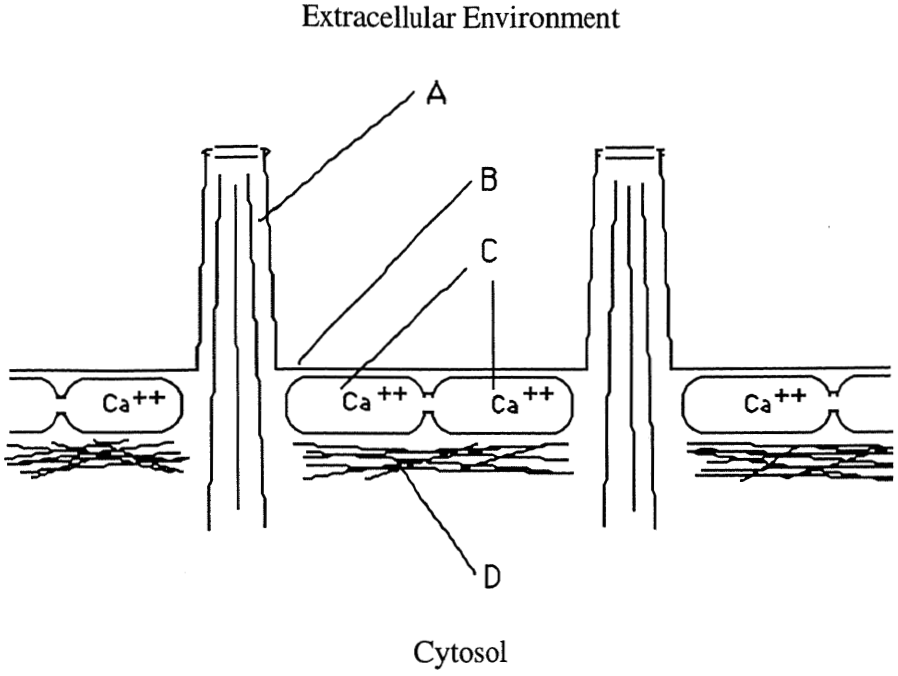


Figure 1. Illustration of the *Tetrahymena* cell pellicle. The cell cortex of *Tetrahymena* has a tripartite structure. Cilia, (A), protrude from the cell cortex into the extracellular environment and are composed largely of microtubules. The cell membrane, (B), is the outermost layer of the pellicle and forms a continuous layer around the cell and cilia. The continuous network of alveolar sacs, (C), lies directly beneath the cell membrane and is interrupted only by cilia. The epiplasm, or membrane skeleton, (D), is composed of filamentous proteins which provide the cell with support and stability as well as a means of controlling the location and distribution of structures along the cell's surface.

molecular weight protein of 23 kDa and small amounts of other proteins (Honts, 1991). Immunofluorescence localization studies using both monoclonal and polyclonal antibodies raised against the three high molecular weight proteins show distinct staining patterns in the cell's epiplasmic layer (Williams *et al.* 1987). Interestingly, monoclonal antibodies produced against bands A, B, and C were monospecific for their respective protein (Williams, 1995), whereas polyclonal antibodies demonstrated that anti-band A was cross-reactive with band B, anti-band B also cross-reactive with band C, and anti-band C was cross-reactive with band B (Williams, 1987).

Under electron microscopy, band C protein in the presence of KCl, *in vitro*, forms a fibrous network of protein similar to spectrin polypeptides that comprise the membrane skeleton of erythrocyte cells (Williams, 1979; Honts, 1991). Spectrin is similar to band C in that it is a relatively large molecular weight protein, it can be separated by low ionic strength from other cortical residues, and some superficial amino acid sequence composition similarities (Honts, 1991). However, band C antibodies failed to recognize spectrin proteins from human red blood cells (Williams *et al.* 1987).

Some low molecular weight proteins isolated from other ciliated protozoa have been shown to be members of the EF-hand Ca^{++}-binding protein family (Vigues and Groliere, 1985; David & Vigues, 1994). Ca^{++} is the most common second messenger in cells and is stored in the alvoelar sacs within the cell cortex. Proteins associated with this family include calmodulin, spasmin, centrin, caltractin, and calmyonemin and all have molecular weights ranging from 20-23 kDa.

In ciliated protozoa, Ca^{++}-binding proteins have been implicated in the regulation of various Ca^{++}-dependent activities. A 22 kDa Ca^{++}-binding protein has been found in the epiplasmic layer of two other ciliated protozoa, *Isotricha* and *Polyplastron*, and is postulated to control the ordering of non-actin microfilaments in the cell cortex (Vigues &

Groliere, 1985). Calmyonemin has been identified in *Eudiplodium magii* (a ciliate) to play a role in regulating myoneme-mediated contraction (David & Vigues, 1994). In the ciliated protozoa *Vorticellid*, spasmin has been shown to be responsible for regulating the contraction of spasmonemes, a contractile organelle present in these organisms (Amos *et. al.* 1975).

Three low molecular weight Ca^{++} binding proteins have been isolated from *Tetrahymena* (Takemasa *et. al.* 1990). These proteins have been designated *Tetrahymena* calcium-binding protein 10, 25 and 23 (or TCBP-10, TCBP-25 and TCBP-23), in accordance with their respective molecular weights (in kDa). Amino acid sequence comparisons indicate that these proteins belong to the EF-hand calcium-binding protein family. Immunofluorescence localization of TCBP-23 has demonstrated it to be found in the cell cortex (Hanyu, 1996). The staining pattern of TCBP-23 polyclonal antibodies exhibits nearly the same characteristics as those seen previously from band C antibodies.

Removal of epiplasmic proteins by a low salt extraction procedure reveals the presence of large amounts of band C protein, as well as smaller amounts of band A and the 23 kDa protein. Polyclonal antisera have been raised against each of these three proteins and have been used to probe Western blots of *Tetrahymena* total cellular protein from whole-cell lysates. Visualization of antibody binding was performed by chemiluminescence and by chloronapthol color development. Whole-cell lysates were prepared both in the presence and absence of protease inhibitors to determine the significance of protein degradation effected by the release of proteases stored in intracellular organelles (Banno and Nozawa, 1982) It has been verified that these three epiplasmic proteins are components of the total cellular protein of *Tetrahymena.* Unexpectedly, it has been demonstrated that the 23 kDa antibodies cross react with a high molecular weight protein.

MATERIALS AND METHODS

Preparation of Low Salt TKI Cortical Extract

Tetrahymena pyriformis strain GL-C cells were grown in 1 L of proteose-peptone media (2% proteose peptone, 1% yeast extract) to mid-logarithmic phase at 28° C (Nelson *et al.* 1981). Low salt extracts of Triton-potassium iodide (TKI) cortical residues were prepared as previously described (Honts, 1991).

Preparation of Antigens for Polyclonal Antibodies

Polyclonal antisera were raised in rabbits against protein band A and band C, and a 23 kDa protein, all of which were extracted from the cell cortex of *Tetrahymena pyriformis* GL. Band A and C proteins were resolved in a 7.5% SDS-polyacrylamide gel while the 23 kDa protein was resolved in a 12.5% SDS-polyacrylamide. This was done to ensure that the three proteins were separated from other proteins found in these low salt TKI cortical extracts. The gels were stained for 30 min in Coomassie Blue R 250 stain (45.4% methanol, 9.2% glacial acetic acid, 45.4% deionized water (ddH_2O), 0.125% Coomassie Blue R-250 Dye and then destained for 30 min in Gel Fixer Destain I (45.4% methanol, 9.2% glacial acetic acid, 45.4% ddH_2O) followed by 30 min in Destain II (5% methanol, 7.5% glacial acetic acid, 87.5% ddH_2O,) (Hoefer, 1997). The gels were then rinsed in ddH_2O and washed overnight in 200 mM phosphate buffer, pH 7.0. The protein bands were excised, quartered and stored at –80° C until injection.

One quarter of each excised band was used per injection. Each gel piece was placed in a Teflon homogenizer with 1 ml of sterile 200 mM phosphate buffer, pH 7.0, and homogenized 20-25 times by grinding the quartered gel band manually (until the gel had been liquefied). For the initial injections, the homogenized protein solution was emulsified

with 1 ml of Freund's Complete Adjuvant (Sigma Chemical Co.) between two glass Micromate syringes 20-25 times. Booster injections were given by emulsifying the homogenized protein solution with 1 ml of Freund's Incomplete Adjuvant (Sigma). All injections were given subcutaneously at two week intervals (Table 1). Injections were given at four sites along the rabbit's spine with approximately 0.5 ml of antigen injected at each site.

The rabbits were bled by one of two methods two weeks after each booster injection. First, blood was collected by placing a 21 gauge needle in the rabbit's central ear vein. If sufficient blood could not be removed by this method, the marginal ear vein was nicked and blood was drawn by placing the ear in a vacuum pump. Approximately 25-40 ml of blood was removed during each bleeding. The serum was obtained by centrifugation of blood samples at 2,310 x g in a Sorvall (model SS-34) rotor for 10 min. Serum samples were stored at –80° C.

Ammonium Sulfate Precipitation

Rabbit γ-globulin fractions of antisera against *Tetrahymena* band A, C, and the 23 kDa protein were obtained by ammonium sulfate precipitation. An equal volume of saturated ammonium sulfate (SAS), pH 7.0, was added to each serum sample slowly over 10 min while stirring on ice. The samples were then allowed to sit for 60 min in an ice bath. The samples were then spun in a Sorvall (model SS-34) rotor at 5,900 x g for 10 min. The pellet was then resuspended in 10 times its volume of 50% SAS and spun in a Sorvall (model SS-34) rotor for 10 min at 5,900 x g. The supernatant was poured off and the pellet was resuspended in 5 ml of 50 mM potassium phosphate buffer, pH 8.0 . The 5 ml γ-globulin fraction was then dialyzed against 50 mM potassium phosphate buffer,

Table 1.

Rabbit Immunization Time Line

Day	Rabbit #1 (band A)	Rabbit #2 (band C)	Rabbit #3 (23 kDa)
Aug 29	Pre-immune bleeding	Pre-immune bleeding	
Sept 3	____	____	
Sept 10	____	____	Pre-immune bleeding
Sept 25	First immunization	First immunization	____
Sept 26	____	____	First immunization
Oct 8	First booster injection	First booster injection	____
Oct 10	____	____	First booster injection
Oct 23	First antisera bleeding	First antisera bleeding	First antisera bleeding
Oct 24	____	____	Second antisera bleeding (Euthanized)
Nov 6	Second booster injection	Second booster injection	____
Nov 20	Second antisera bleeding	____	____
Nov 24	____	Second antisera bleeding	____
Mar 18	Third booster injection	____	____
Mar 19	____	Third booster injection	____
Apr 2	Third antisera bleeding		____
Apr 3	____	Third antisera bleeding	____
Apr 9	Fourth antisera bleeding (Euthanized)		____
Apr 10		Fourth antisera bleeding (Euthanized)	____

pH 8.0, for 24 hours at 4° C. The γ-globulin fractions for each antisera were then stored at –80° C.

Ion-Exchange Chromatography of γ globulin Antisera

Rabbit IgG antibody samples against *Tetrahymena* band A, C, and the 23 kDa protein, were separated by ion-exchange chromatography from the γ-globulin antisera fractions. Two ml of packed DEAE Sepharose CL 6B resin (Sigma) were placed in separate 15 ml disposable columns for each γ globulin fraction. The resin was washed with 20 ml of 5 mM potassium phosphate buffer, pH 8.0, and the elution flow rate was adjusted to 1 ml/min. The γ-globulin fraction was placed on the column, allowed to run through, and the eluate was collected. The column was then washed with 10 ml of 5 mM potassium phosphate buffer, pH 8.0, and the eluate was collected. The wash was repeated once. A_{280} measurements were taken on the three eluted samples to verify that the IgG antibodies were eluted in the first two samples. The column was then washed with 10 ml of 300 mM potassium phosphate buffer, pH 8.0, buffer twice to remove IgM antibodies and other bound serum proteins and the eluates were collected. All collected fractions were stored at –80° C.

Affinity Purification of IgG Polyclonal Antibodies

Affinity purification was performed on the IgG antibody fractions of *Tetrahymena* bands A, C, and the 23 kDa protein. 200 µl samples of low salt extracts from TKI cortical residues (enriched in band A, C, and 23 kDa proteins) were run in two 7.5% SDS-polyacrylamide gels to separate bands A and C from other cortical proteins. Two 12.5% SDS-polyacrylamide gels were loaded with 200 µl of the same low salt extract from TKI cortical residues to resolve the 23 kDa protein.

The proteins were then blotted onto nitrocellulose using an electrophoretic transfer apparatus (TE Series Transphor Unit, Hoefer) at 50 V, 200 mA, and 3 W. A modified Towbin transfer buffer (0.250 M Tris, 0.192 M glycine) was used in the transfer, according to Anderson *et al*, 1982. Band A and C proteins were transferred for 20 hours and the 23 kDa protein was transferred for 8 hours. In order to estimate the completeness of protein transfer, the gels were stained in Coomasie Blue R-250 and destained as before, after electrophoretic transfer. Complete transfer of all low molecular weight proteins was observed after 8 hrs and near complete transfer of high molecular weight proteins was observed after 20 hours. The nitrocellulose strips were stained in Ponceau S stain (5% glacial acetic acid and 0.1% Ponceau S powder) for five min. The stained protein bands were then excised. The stain was removed from the excised strips by washing twice in ddH_2O, once in a basic solution of 1 M NaOH, and twice more in ddH_2O. The strips were blocked for 1 hr in BLOTTO (5% nonfat dry milk in Tris-buffered saline).

The strips were placed in their respective IgG purified antibody solutions and incubated for 2 hr. The strips were then washed in 1 ml of 0.2 M glycine-HCl pH 2.5 for five minutes to remove bound antibodies. The pH of the extract was then raised by adding 0.2 M Tris-HCl, pH 8.0, as described by Goers (1993). This extraction procedure was repeated twice. The extracts containing the affinity-purified antibodies were pooled and concentrated by centrifugation in a Sorvall (model SS-34) rotor using Centricon C30 microconcentrators at 2,310 x g for 10 min. The concentrated, affinity-purified antibodies were stored at –80° C

Preparation of Total Cell Protein Samples

Tetrahymena pyriformis GL cells were grown in 200 ml proteose-peptone media at 28° C until the cells had reached mid-logarithmic phase (Nelsen *et al.* 1981).

Mid-logarithmic phase was determined by taking the absorbance of the culture at 600 nm every 5 hours during lag phase and every 2 hours afterwards (Table 2, Graph 1). The cells were then harvested by spinning 1 ml of the cell culture, in a 1.5 ml microcentrifuge tube, in a Fisher Scientific (Marathon 26km) microcentrifuge at 600 x g for 10 min to pellet the cells. The cells were then lysed by mixing the pellet with 1 ml of boiling Laemmli SDS-sampling buffer (Laemmli, 1970) and vortexing to resolubilize the pellet. The sample was then boiled for 5 min and stored at –80° C.

Proteolytic inhibitors were also employed to prevent degradation of total cellular proteins during cell lysis by the release of proteases stored within intracellular compartments. Laemmli SDS-sample buffer containing 0.1 mM EDTA, 0.1 mM EGTA, and 10 μM leupeptin was prepared. Cells were pelleted, as before, and three test were performed. Method 1: A cell pellet was boiled for 5 min and then mixed with 1 ml of room temperature Laemmli SDS-sampling buffer containing the protease inhibitor cocktail. The pellet was then resolublized on a vortexer and boiled for 5 min. Method 2: A pellet was mixed with 1 ml of room temperature Laemmli SDS-sampling buffer containing the protease inhibitor cocktail. The pellet was resolublized by vortexing and the sample was boiled for 5 min. Method 3: A pellet was mixed with 1 ml of boiling Laemmli SDS-sampling buffer containing the protease inhibitor cocktail. The pellet was resolublized by vortexing and then boiled for 5 min. The samples were resolved in 10% SDS-polyacrylamide gel and stained with Coomasie Blue R-250 and destained with Destain I & II, as before.

Immunoblotting Analysis

SDS-PAGE was performed on low salt extracts from TKI cortical residues and on all total cellular protein preparations from whole-cell lysates prepared with and without

Table 2.

Determination of *Tetrahymena* Mid-logarithmic Growth Phase

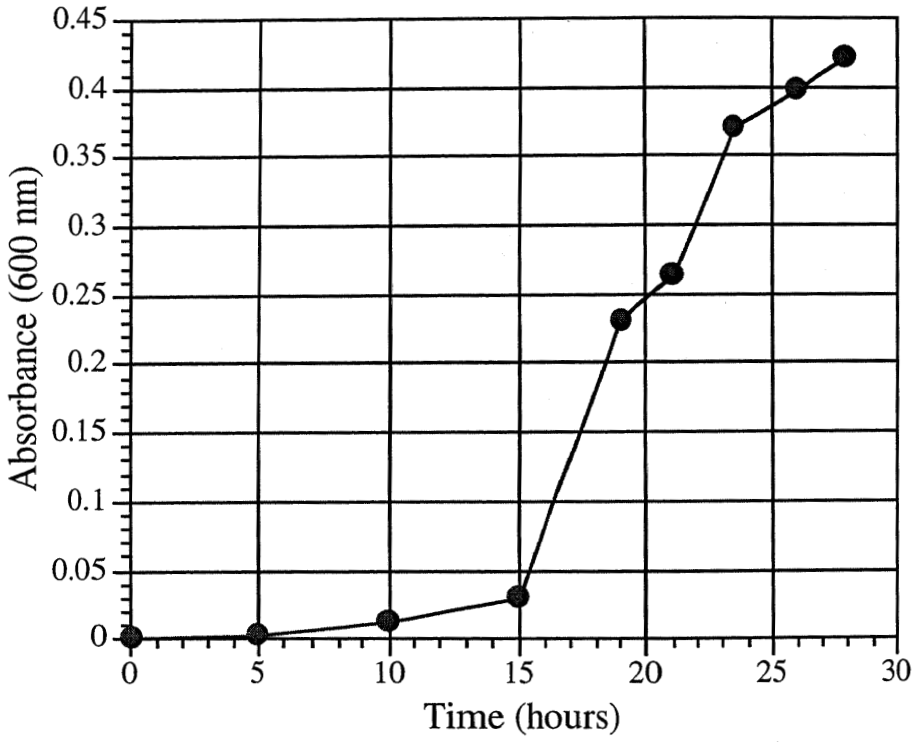
(Absorbance of *Tetrahymena* Cell Cultures at 600 nm)

Time (hours)	Absorbance
0	0.000
5	0.002
10	0.012
15	0.030
19	0.231
21	0.264
23.5	0.371
26	0.397
28	0.421

Note: 200 ml PPY media inoculated with a 5 ml of *Tetrahymena* cell culture

Graph 1.

Growth Curve of Tetrahymena pyriformis GL
(Absorbance vs. Time)



proteases inhibitors. The samples were resolved in a 10% polyacrylamide gel. Extracts were then transferred to nitrocellulose using an electrophoretic transfer apparatus (TE Series Transphor Unit, Hoefer), at 50 V, 200 mA, and 3 W, and the modified Towbin transfer buffer according to Anderson *et al*, 1982. The blots were nonspecifically blocked with BLOTTO (5% nonfat dry milk in Tris-buffered saline) for 1 hr. The strips were then probed with a 1:25 dilution of affinity purified band C antibody or a 1:750 dilution of band A and 23 kDa whole sera in BLOTTO for 1 hr. They were then washed for 30 min with BLOTTO. The strips were then probed with a 1:5000 dilution of peroxidase labeled anti-rabbit IgG antibodies in BLOTTO. The strips were then washed for 30 min with BLOTTO. All incubations were performed at room temperature.

Visualization of antibody bands was performed and developed by one of two methods. In some cases, color development by chloronapthol was performed. The strips were placed in a chloronapthol color development solution (0.3 ml 3% H_2O_2, 50 ml of 0.05 M phosphate buffer pH 6.5, and 30 mg of 4-chloro-1-napthol dissolved in 10 ml of ice cold methanol). Blots were exposed to chloronapthol for 1 hr. In other cases, chemiluminescence was used to develop immunoblots by incubating strips in 20 ml of ECL Western Blotting Reagant I and II (Amersham) in a 1:1 ratio for 5 min. The immunoblots were then exposed to Kodak Scientific Imaging Film (Biomax ML). Exposure times varied and several exposures were performed for each immunoblot ranging from 10 sec to 5 min.

RESULTS

Analysis of Low Salt TKI Cortical Extracts

By using low salt extracts of TKI cortical residues, the three epiplasmic proteins (bands A, C, and a 23 kDa protein) can be partially purified (Honts, 1991). These TKI cortical extracts were analyzed by SDS-PAGE in a 10% gel (Fig 2). Protein samples obtained by this method were enriched in band C protein (125 kDa), as well as lesser amounts of band A (235 kDa) and a 23 kDa protein. This method has been demonstrated to be successful at separating these proteins from other membrane skeletal components (such as band B and tetrins).

Total Cellular Protein Preparation from Whole-Cell Lysates

A protease inhibitor cocktail was employed to determine the significance of proteolytic activity by the release of enzymes from intracellular storage organelles during cell lysis. *Tetrahymena* whole-cell lysates were visualized by 10% SDS-polyacrylamide gel electrophoresis in the presence of protease inhibitors and without inhibitors (Fig. 3). Subtle differences were demonstrated between protein staining patterns of fractions containing the protease inhibitor cocktail compared to staining patterns of fractions without protease inhibitors (lane 2 vs. lane 4 and 5). Differences between both high and low molecular weight proteins were evident. These unique bands present different total cellular antigens to be probed during immunoblots.

The release of intracellular proteases during cell lysis has been demonstrated to promote proteolysis of cellular proteins (Hirono *et al.* 1989) and this must be considered during further investigations. The gel staining pattern for cells lysed in the presence of the

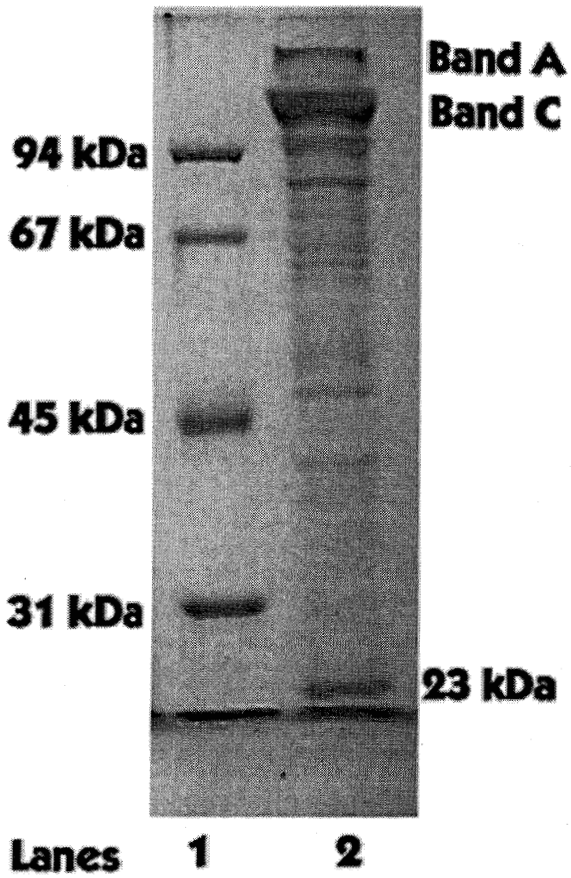


Fig. 2. SDS-PAGE analysis of low salt extracts of TKI cortical residues from *Tetrahymena pyriformis* GL in a 10% SDS-polyacrylamide gel. This particular preparation was used to prepare antigens for injections . Lane 1 contains molecular weight markers purchased from Bio-Rad. Lane 2 contains extracts enriched in band C protein (125 kDa), as well as lesser amounts of band A protein (235 kDa) and a 23 kDa protein.

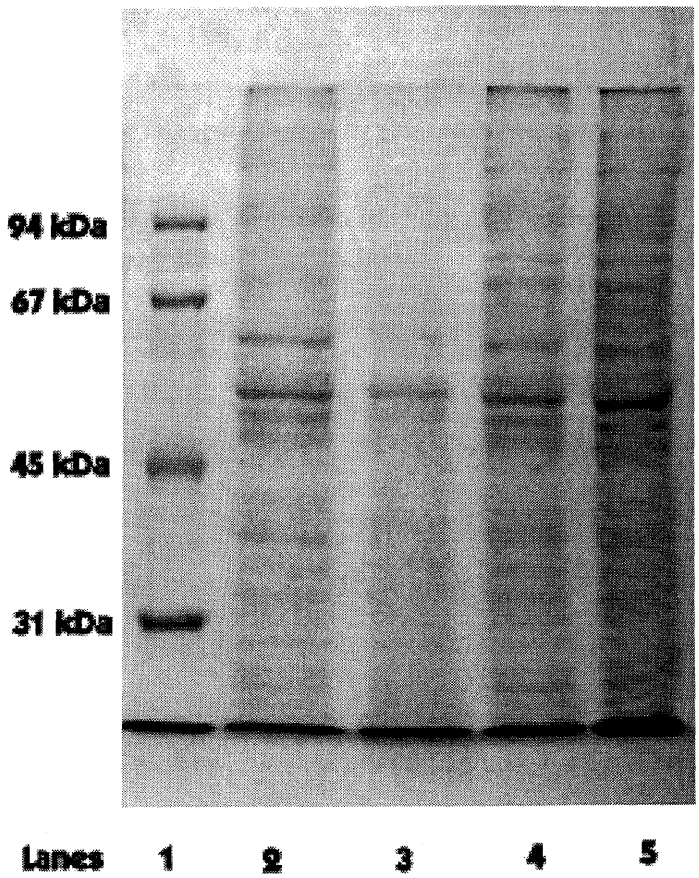


Fig. 3. SDS-PAGE analysis of total cellular protein from Tetrahymena *pyriformis* GL. Samples with and without the presence of protease inhibitors in a 10% SDS-polyacrylamide gel. Lane 1 contains molecular weight markers (Bio-Rad). Lane 2 contains total cellular protein prepared without protease inhibitors. Lane 3 contains total cellular protein obtained by boiling pellet followed by adding room temperature Laemmli sample buffer with protease inhibitors and boiling again (method 1). Lane 4 contains total cellular protein obtained by adding room temperature Laemmli sample buffer with protease inhibitors to cells followed by boiling (method 2). Lane 5 contains total cellular protein obtained by adding boiling Laemmli sample buffer with protease inhibitors to cells followed by boiling (method 3).

protease inhibitor cocktail in Laemmli sample buffer prior to lysis (lanes 4 and 5) were demonstrated to be the same. Preparation of whole cell lysates by boiling the pellet (method 1), followed by the addition of the protease inhibitor cocktail in Laemmli sample buffer (lane 3) was shown to be insufficient in generating high protein concentrations.

Characterization of Polyclonal Antibodies

Polyclonal antibodies were produced in rabbits against three *Tetrahymena* membrane skeletal proteins purified from cortical residues by low salt extraction. Low salt extracts of TKI cortical residues and total cellular protein derived from whole-cell lysates were examined by immunoblotting to demonstrate the presence of these proteins as major components of total cellular protein. Rabbit pre-immune sera did not detect bands from *Tetrahymena* total cellular protein or TKI cortical residues (not shown).

Attempts to affinity-purify polyclonal antibodies against band A were unsuccessful. Therefore, polyclonal antibodies from whole serum against band A protein were used. These antibodies did not recognize a protein band of 235 kDa corresponding to the molecular weight of band A (Fig. 4). Visualization was done by chloronapthol color development. However, band A antibodies from whole sera recognized three bands of between 100 kDa and 200 kDa (lane 2).

Affinity-purified antibodies against band C were used to probe total cellular protein and TKI cortical extracts (Fig. 5). Affinity-purified polyclonal antibodies against band C protein recognized a 125 kDa protein from low salt extracts of TKI cortical residues, which are highly enriched in band C, as well as several smaller molecular weight bands when visualized by chemiluminescence (lane 3). These bands may be the result of proteolytic degradation of band C. Affinity-purified band C antibodies apparently recognized a single 125 kDa band exclusively (lanes 4 and 8) from all but one preparation (lane 6) of

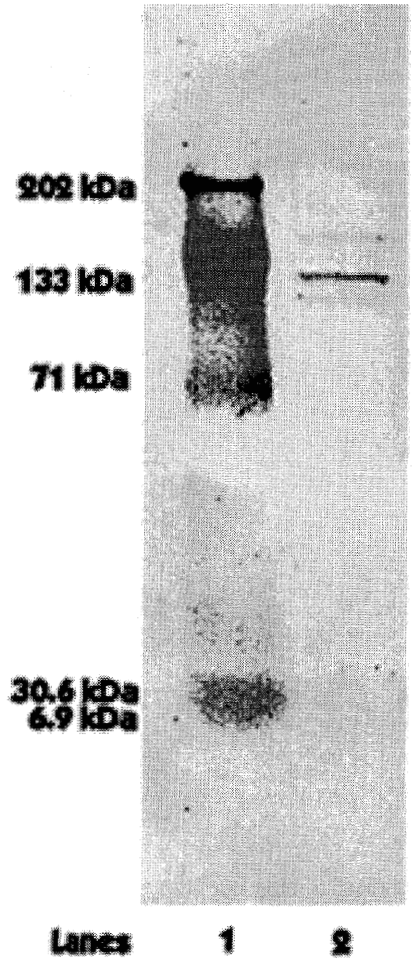


Fig. 4. Immunoblots using anti-A polyclonal antibody serum. Lane 1 contains molecular weight markers (Bio-Rad). Lane 2 contains anti-A polyclonal antibody serum probed against total cellular protein prepared without protease inhibitors. Anti-A dilutions were at 1:750. Blot developed by chloronapthol.

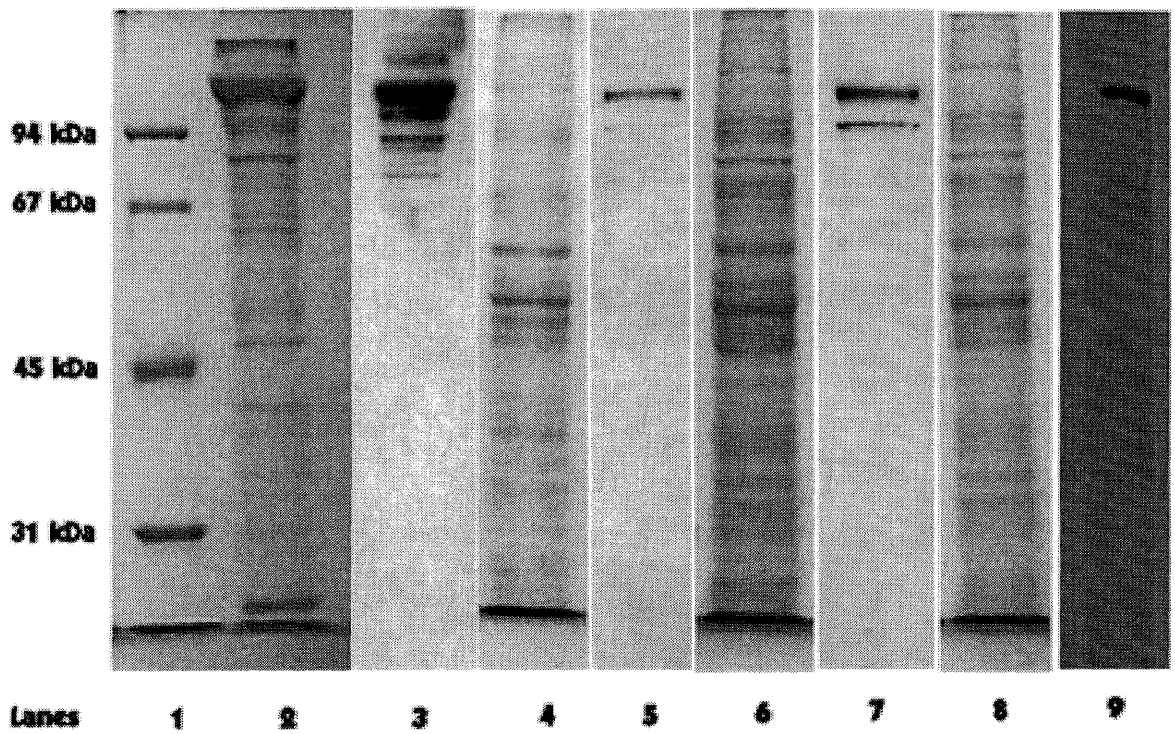


Fig. 5. Immunoblots of affinity-purified anti-C polyclonal antibodies. Both TKI cortical residues and *Tetrahymena* total cellular protein with and without the presence of protease inhibitors were probed by anti-C antibodies. Lane 1 contains molecular weight markers (Bio-Rad). Lane 2 contains low salt extractions of TKI cortical residues (rich in band C). Lane 3 contains anti-C antibodies probed against low salt extractions of TKI cortical residues. Lane 4 contains total cellular protein prepared without protease inhibitors. Lane 5 contains anti-C antibodies probed against total cellular protein preparation in lane 4. Lane 6 contains total cellular protein preparation prepared with protease inhibitor cocktail method #2. Lane 7 contains anti-C antibodies probed against total cellular protein preparation in lane 6. Lane 8 contains total cellular protein preparation prepared by protease inhibitor method #3. Lane 9 contains anti-C antibodies probed against total cellular protein preparation in lane 8. Anti-C antibody dilutions were at 1:25. Immunoblots developed by chemiluminescence.

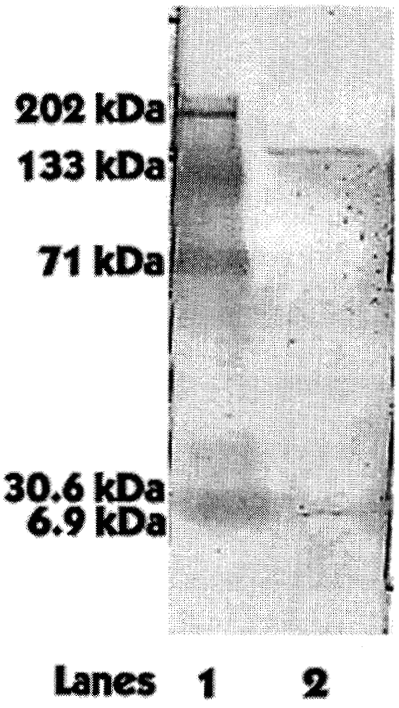


Fig. 6. Immunoblots using anti-23 kDa polyclonal antibody serum. Lane 1 contains molecular weight markers (Bio-Rad). Lane 2 contains anti-23 kDa polyclonal antibody serum probed against total cellular protein prepared without protease inhibitors. Anti-23 kDa dilution at 1:750. Immunoblot developed by chloronapthol.

Tetrahymena total cellular protein. Two bands were detected by band C antibodies probed against total cellular protein prepared by the protease inhibitor method #2 prep (lane 6). This is a surprising result, since this method involved the use of protease inhibitors to prevent proteolysis. It is possible that this method preserved a cross-reactive antigen that is degraded by other preparation methods.

An attempt was made to strip the primary and secondary antibodies from total cellular proteins bound to nitrocellulose used in the band C immunoblots. A 0.2 M glycine-HCl pH 2.5 solution was used to remove the antibodies. Visualization of the nitrocellulose was done by Ponceau S staining to determine if the band C antibodies and the anti-rabbit IgG antibodies were removed. The presence of a single band at 125 kDa demonstrated that the antibodies were irreversibly bound to the nitrocellulose after several days storage at 4° C. This is a surprising result, since the same 0.2 M glycine-HCl solution was used to remove antibodies from nitrocellulose during the affinity purification procedure. However, during this procedure the immunoblots had been subjected to a secondary antibody containing a peroxidase label, a substrate to develop the immunoblots, and were stored for several days at 4° C. Any of these factors may have affected the irreversible binding of band C antibodies.

An attempt to affinity-purify the antibody directed against the 23 kDa protein was unsuccessful. Therefore, polyclonal antibodies from whole serum against the 23 kDa protein were used. Interestingly, polyclonal antibodies against the 23 kDa protein recognized both a 23 kDa protein and a high molecular weight protein above 125 kDA from *Tetrahymena* total cellular protein.

DISCUSSION

The preparation of new polyclonal antibodies against band A, band C, and the 23 kDa protein provided a means by which previously conflicting results could be reanalyzed. Previously, monoclonal antibodies raised against bands A, B, and C were monospecific for their respective proteins. However, polyclonal antibodies raised against bands A, B, and C showed immunological cross-reactivity exists between the proteins. Band A antibodies recognized bands A and B, band B antibodies recognized bands B and C, and band C antibodies recognized bands C and B.

In this study, affinity-purified polyclonal antibodies against band C were used to probe TKI cortical extracts and total cellular protein derived from whole-cell lysates. The new polyclonal band C antibodies recognized only band C protein (125 kDa) or smaller molecular weight proteins and were not cross-reactive with band B, as seen before (Williams, 1987). Previous studies demonstrated that few similarities exist between digested fragments of band C and B in peptide maps, although a striking similarity exists between the two proteins' amino acid composition (Honts, 1991). Therefore, the results of previous studies employing monoclonal antibodies are supported by the present data.

Polyclonal antibodies against band A protein were not able to recognize the 235 kDa protein that corresponds to the apparent molecular weight of band A. However, the antibodies did recognize three bands at a lower molecular weight ranging between 100-200 kDa. These initial result may support previous results found with polyclonal band A antibodies which were immunologically cross-reactive with band B (145 kDa), but further tests must be done to confirm these findings.

Polyclonal antibodies raised against the 23 kDa protein revealed an interesting result. Both a 23 kDa protein and a high molecular weight protein above 125 kDa were stained. The exact molecular weight of this high molecular weight protein is unclear at this

time. Possible explanations for this unusual finding may be that the 23 kDa protein and a high molecular weight protein are from the same protein family. Therefore, the two proteins would express similar epitopes which are recognized by the 23 kDa antibodies.

A second explanation maybe that a high molecular weight protein is tightly associated with the 23 kDa protein or possibly that several 23 kDa subunits are associated to form a large oligomer. Although SDS was employed, which serves to dissociate tightly associated proteins such as dimers and oligomers, it is possible that the high molecular weight protein may contain polypeptide subunits which do not dissociate into its subunits in the presence of SDS. The inability of proteins to dissociate has been demonstrated by band C complexes termed "super A bands" which fail to dissociate in SDS-sampling buffers with Tris buffers less than pH 9.0 (Honts, 1991). This may serve as an explanation for the 23 kDa protein recognizing a high molecular weight protein. Further research is needed to fully understand the relationship between the 23 kDa protein and the high molecular weight protein.

The epiplasmic layer of *Tetrahymena* likely serves the same function as that found in other ciliates, such as *Paramecium* and *Pseudomicrothorax*, and other protists such as euglenas and dinoflagellates. In all these organisms, the epiplasmic layer provides strength and rigidity to the cell surface as well as providing a possible means for the regulation and distribution of structures along the cell's surface. *In vitro* studies of this layer, under electron microscopy, reveal the presence of a filamentous protein network (Huttenlauch *et al.* 1995). However, the composition of proteins that constitute the epiplasmic layer varies greatly between species and common principles of epiplasmic construction have yet to emerge.

The study of the ciliated protozoa *Tetrahymena* provides a simple and basic model for understanding protein interactions associated with the epiplasm compared to that found

in other ciliates. The epiplasm of *Tetrahymena* is composed largely of three high molecular weight proteins, bands A, B, and C. The epiplasm of *Paramecium* is composed of 30-50 protein bands of differing molecular weights. These proteins are referred to as epiplasmins and have been shown under electron microscopy to form filamentous aggregates, *in vitro* (Coffe *et al.* 1996). The epiplasm of *Pseudomicrothorax dubius* (a ciliate) consists of two major groups of proteins with apparent molecular weights between 78-80 kDa and 11-13 kDa. *In vitro*, electron micrographs of these proteins reveals the formation of short filamentous polymers which congregate to create thick filaments, sheets, or tubes (Huttenlauch *et al.* 1995). Given the complexity of these protein structures in other ciliates, *Tetrahymena* is an ideal organism for studying epiplasmic protein associations.

FUTURE RESEARCH

The results presented in this paper lay the ground work for many future research projects. First, the affinity purification of band A and the 23 kDa antibodies must be completed. Affinity-purified band A antibodies will be used to confirm the results of whether band A is cross-reactive with band B, or any other protein from whole-cell lysates, as previously demonstrated by polyclonal antibodies.

Affinity purified 23 kDa antibodies will be useful in studying the cross-reactivity anti-23 kDa antibodies had with a high molecular weight protein from total cellular protein. An exact molecular weight of the cross-reactive high molecular weight protein with anti-23 kDa will also need to be determined. Additionally, more work needs to be done to determine whether the 23 kDa protein is the same as the Ca^{++}-binding protein, TCBP-23, characterized by Hirono et al. (1989). Since TCBP-23 was shown to be located in the same

region of the epiplasm as band C by immunofluorescence, it would be of interest to understand what role, if any, this protein plays in the formation of epiplasmic protein filaments.

The production of these three polyclonal antibodies will provide a means by which investigators can study the interactions between membrane skeletal proteins in *Tetrahymena.* These antibodies will be useful in studying the composition, formation, and possible regulation of epiplasmic protein filaments. These filaments are thought to not only be responsible for maintaining cell shape and stability, but providing a means by which the distribution of structures on the cell's surface may be regulated during cellular development.

Furthermore, they will provide a means by which the membrane skeleton of *Tetrahymena* can be compared with the cell cortexes of other ciliates and mammalian cells. Although bands A, B, and C seem to be unique proteins to *Tetrahymena,* information on their composition, structure, and function maybe useful in studying and understanding the roles of cytoskeletal proteins in all cells.

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